

DOCKET NO.: SNL-0004 / SD-8433
Application No.: 10/701,097
Office Action Dated: October 25, 2007

PATENT

In re Application of:
Jason A.A. West, et al.

Confirmation No.: **4414**

Application No.: **10/701,097**

Group Art Unit: **1744**

Filing Date: **November 4, 2003**

Examiner: **Bowers, Nathan Andrew**

For: **MICROFLUIDIC INTEGRATED MICROARRAYS FOR BIOLOGICAL
DETECTION**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION OF DR. JASON A.A. WEST UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Jason A.A. West, Ph.D., hereby declare the following:

1. I am a co-inventor of the above-captioned patent application ("the subject application"). My Curriculum Vitae is enclosed as **Exhibit A**. Among my credentials, I earned a B.Sc. in Toxicology and Chemistry from the University of Massachusetts, Amherst, USA and a Ph.D. in Pharmacology and Toxicology from the University of California, Davis, USA. I am a co-founder of Arcxis Biotechnologies of Pleasanton, California, USA, and am presently the Chief Technology Officer of that company.
2. I have performed microfluidics work relevant to the work described in this patent application as a post-doctoral fellow at the Lawrence Livermore National Laboratories, where I worked in close collaboration with General Electric Healthcare (formerly Molecular Dynamics) a microarray company. I continued this work as a Senior member of the Technical Staff at Sandia National Laboratories in the Microfluidics Research Department,

which group was a worldwide leader in the field of microfluidics. After completion of various microfluidics prototype systems, Arcxis Biotechnologies was founded to move the research into the product phase.

3. It is my understanding that claims 1-114 of the subject application are directed toward microfluidic chips.

4. I have reviewed the Office Action dated January 3, 2008. As I understand it, the Examiner has rejected claim 1 and those claims that depend from claim 1 for allegedly being obvious in light of the prior art, in particular U.S. Application No. 2004/0209354 to Mathies, U.S. Application No. 2004/0037739 to McNeely; and U.S. Pat. No. 6,833,242 to Quake.

5. This declaration is made to demonstrate that microfluidic chips recited in claim 1 of the subject application were not obvious at the time that the application was filed.

6. Based on my experience in the field of microfluidics and my own observations and tests, the invention of the instant application achieves results that are unexpectedly superior to those of alternative devices in the field.

7. Oligonucleotides processed by the claimed invention achieved in only several minutes a level of hybridization to target probes that oligonucleotides processed by alternative products required several hours to achieve. This is shown in the attached **Exhibit B**, which compares the performance of the invention that is the subject of this patent application with an existing alternative product. Also attached, as **Exhibit C**, is a manuscript that demonstrates the ability of the invention that is the subject of this patent application to perform hybridizations in only twenty minutes. In my view, this performance would not have been predicted by one of ordinary skill in the art at the time of invention.

8. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that

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these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

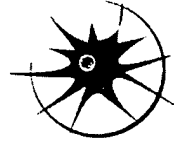
Dated: March 25, 2008

/Jason A.A. West, Ph.D./
Jason A.A. West, Ph.D.

Attachments:

Exhibit A
Exhibit B
Exhibit C

EXHIBIT A



Experience:

2003-Present **Founder**

Chief Technology Officer, Sr. Director of Research and Development
Arcxis Biotechnologies, Pleasanton, CA. 94566

Principal investigator: Phase I/II SBIR, BioPhalanx™, Hand-held instrument and consumable platform for biological agent detection.

Principal investigator: Commercial development of the Lysix™ Platform for automated nucleic acid and protein purification.

Principal investigator: Development of Altix™ Platform, a microfluidic integrated microarray system including instrumentation.

Principal investigator: Tentacle Probes™, molecular diagnostic reagents.

IP manager: Preparation and filing of Patents and Freedom to Operate opinions

Fundraising: Completed Seed round, grant funding and Series A/A1 financing.

2001-2005

Senior Member Technical Staff

Sandia National Laboratory, Livermore, CA. 94551-0969

Principal investigator: Design and fabrication of a microfluidic device integrated with a gene arrays for the detection of genetically engineered biological agents.

Principal investigator: Development of an unattended water sensor (UWS) for protein toxins and bacteria in municipal water supplies.

Team Leader: Directing development of Sandia's µChemlab project, focused on the detection of biological toxins and pathogenic agents.

2000-2001

Post-Doctoral Scientist

Lawrence Livermore National Laboratory, Livermore CA. 94550

Coordinate studies using AMS and Microarrays. Chemoprevention of Prostate Cancer; Studies based on gene expression patterns after DNA damage and alterations metabolic activation of pro-carcinogens in rats and humans.

1995-2000

Research assistant

University of California, Davis, School of Veterinary Medicine: APC

Specialization: Development of metabolic tolerance in response to repeated acute xenobiotic exposure

1994-1995

Staff Scientist

Energy and Environmental Engineering, Somerville, MA 02143

Duties: R&D- HPLC method development for quantitative analysis of soil/water/TCLP matrices for phenoxy acid herbicide and explosives content.

1992-1994

Laboratory Technician

Massachusetts Pesticide Analysis Laboratory, Amherst, MA 01003

Duties: Matrix extraction, sample preparation, GC, HPLC, analysis of pesticide and herbicide/pesticide residues.

Technical Skills:

- Analytical: GC, HPLC, IEC, spectrophotometric, spectrofluorometric, atomic absorption spectrophotometric analysis; Method development for analysis of mammalian drug/toxicant and cellular metabolism processes, including use of radioactive compounds; Analysis of chemical residues in environmental samples, including herbicides, pesticides, and explosives; Peptide chromatography, Capillary electrophoresis, microfluidics, photo-castable polymer chemistry.
- Mol. Bio.: Microarray analysis, real-time PCR, Protein/nucleic acid extraction, isolation, and blotting, protein activity measurement, transport assay development, primary cell isolation and culture, bacterial culture, drug metabolism analysis, tissue microdissection.
- Microscopy: Epifluorescent and confocal microscopy, light microscopy, high-resolution histopathology, immunohistochemistry and cytochemistry, scanning electron microscopy, *In Situ* PCR, semi-quantitative fluorescent image analysis.
- Computer: Apple and IBM Platforms, Web page design and construction, comprehensive use of various imaging and graphics design programs. Extensive use of Microsoft Office suite as well as MS Project, MS Visio. working knowledge of Solidworks and Alibre CAD modeling software.

Education

- Doctorate: Ph.D. Pharmacology and Toxicology (2000)
Pharmacology and Toxicology Graduate Group
University of California, Davis, CA 95616
- Dissertation:** "Shifts in Clara Cell Susceptibility to Chemical Mediated Injury: Mechanistic Studies of Phase II Metabolic Processes Resulting in Tolerance to Bioactivated Toxicants"
- Undergraduate: BS Environmental Science (1994)
Minor: Chemistry
Department of Environmental Science
University of Massachusetts, Amherst, MA 01003

Ongoing and former projects:

- Development of real-time PCR reagent platform (Tentacle Probes) for infectious disease testing, including HSV I/II, Sepsis, and bioterror agents.
- Commercialization of an integrated sample preparation technologies (Lysix™), and a microfluidic microarray platform (Altix™).
- Principal Investigator for the the Phase I and II SBIR, BioPhalanx™, an Integrated diagnostic Platform for infectious disease and biological weapons.
- Development of a microfluidic integrated microarray for the detection of genetically engineered biological agents.
- Optimization of microchannel electrophoretic protein separation and detection of biotoxins on microfluidic devices.
- Development and optimization of μ Chemlab methods for the detection and identification of bacterial and viral agents.

Collaborations:

- Kaiser Permanente Clinical Diagnostic Laboratory (2006- Present)
- California Department of Health, Viral Disease and Rickettsia laboratory (2005-Present)
- Arizona State University Harrington, Department of Bioengineering (2005-present)
- Sandia National laboratories (2005- Present)
- United States Army Research Institute of Infectious Disease (2003-present)
- University of California Davis, Microarray Center (2003-present)

Professional Affiliations:

- Society of Toxicology
- American Society of Pharmacology and Experimental Therapeutics

Awards/Fellowship:

- Employee recognition award – μ ChemLab development, Sandia National Laboratories - 2003
- Award of excellence - μ ChemLab development, Sandia National Laboratories - 2003
- NIEHS Superfund fellowship (1999)
- Ralph Kittell Fellowship – Innovative teaching project (1998)
- Jastro Shields Fellowship – Academic (1996/1997)

Extracurricular activities:

- Valley Spokesman Racing Team (cycling: 2000-present)
- Team in Training- a Leukemia Society of America Fundraiser
- Rowing coach – University of California, Davis men's varsity crew (1995-2000)

Peer-Reviewed Publications:

- Brent C. Satterfield^{a,h}, Matt Bartosiewicz^a, **Jay A.A. West^a** (corresponding author) (2008) *Revolutionizing detection and typing of polymorphic organisms and G-T differentiation through the use of cooperative probe technologies* (manuscript in preparation)
- Brent C. Satterfield, Matt Bartosiewicz, **Jay A.A. West*** (corresponding author), Michael R. Caplan. (2008) *Surpassing specificity limits of nucleic acid probes via cooperativity* (manuscript in preparation)
- Brent C. Satterfield^a, Matt Bartosiewicz^a, Ivy C. Yeung^b, Mark J. Stanley^b, **Jay A.A. West^{a*}** (corresponding author) (2008) *Improving typing of polymorphic organisms through the use of cooperative probe technologies*(manuscript in preparation)
- Brent C. Satterfield, Michael R. Caplan*(corresponding author), **Jay A. A. West** (2008) *Tentacle Probe sandwich assay in porous polymer monolith improves specificity, sensitivity and kinetics.* (Accepted with revisions at Nucleic Acids Research)
- Brent C. Satterfield, David A. Kulesh, David A. Norwood, Leonard P. Wasieloski, Jr, Michael R. Caplan^a, **Jay A.A. West***, (2007). "Tentacle Probes: differentiation of difficult single-nucleotide polymorphisms and deletions by presence or absence of a signal in real-time PCR." *Clin Chem* **53**(12): 2042-50.
- Brent C. Satterfield, Seth Stern, Michael R. Caplan^b, Kyle W. Hukari^a, and **Jay A.A. West*** (corresponding author) (2007). "Microfluidic purification and preconcentration of mRNA by flow-through polymeric monolith." *Anal Chem* **79**(16): 6230-5.

- Brent C. Satterfield, **Jay A.A. West*** (corresponding author), Michael R. Caplan, *Tentacle probes: eliminating false positives without sacrificing sensitivity*. Nucleic Acids Research, 2007, Vol. 35, No. 10
- **Jay A. A. West and Brent C. Satterfield**. *Fabrication of Porous Polymer Monoliths in Microfluidic Chips for Selective Nucleic Acid Concentration and Purification. Chapter 2*, Microchip-based assay systems : methods and applications. Humana Press, Editor: Pierre N. Floriano, 2007.
- **Jay A.A. West** Kyle W. Hukari, Kamlesh Patel, Ronald F. Renzi,. Rapid Universal Solubilization and Analysis of Viruses and Bacteria using a Simple Flow-Through Ultra-High Temperature Capillary Sample Preparation Device. - 2008 – *manuscript in preparation to Biochemical and Biophysical research communication*.
- Ronald Renzi, James Stamps, Victoria VanderNoot, Brent Horn, **Jay A.A. West**, Boyd J. Wiendenman, Robert Crocker, Scott Ferko, and Julia Fruetel. Hand-Portable Micro Analytical Instrument for Reusable Chip-Based Electrophoresis. Part 1: System Design and Integration - 2005 – Analytical Chemistry 77: 435-441.
- Julia A. Fruetel, Victoria VanderNoot, Brent A Horn, **Jay A.A. West**, Ronald Renzi, Scott Ferko, James F. Stamps, Isaac R. Shokair, Daniel Yee, Robert Crocker, Boyd Wiedenman. "Chip based analysis of protein biotoxins integrated into a hand portable device" – 2005 - Electrophoresis 26(6): 1144-54.
- **West, Jay A.A.**, Laura S. Van Winkle, Dexter Morin, Chad A. Fleschner, Henry Jay Forman, Alan R. Buckpitt, and Charles G. Plopper. Repeated Inhalation Exposures of the Bioactivated Cytotoxicant Naphthalene (NA) Produce Airway Specific Clara Cell Tolerance in Mice **Cover page** (2003) *Toxicological Sciences*. 75 (1) 161-168.
- Williams, KJ; **West, JAA**; Fleschner, CA; Plopper, CG. Airway Epithelial Stress Protein Expression During Repair After Acute Clara Cell Loss and in the Development of Tolerance. 2003 submitted to *Toxicology and Applied Pharmacology* (accepted).
- Buckpitt, A., Boland, B., Isbell, M., Morin, D., Shultz, M., Baldwin, R., Chan, K., Karlsson, A. Lin, C., Taff, A., **West, J.**, Fanucchi, M., Van Winkle, L., Plopper C.G. Naphthalene-induced respiratory tract toxicity: Metabolic mechanisms of toxicity. *Drug Metabolism Reviews*. -2002- v.34, no.4, p.791-820
- **West, Jay A.A.**, Kurt J. Williams, Elina Toskala, Susan J. Nishio, Chad A. Fleschner, Henry Jay Forman, Alan R. Buckpitt and Charles G. Plopper. (2002) Induction of Tolerance to Naphthalene in Clara Cells is Dependent on a Stable Phenotypic Adaptation Favoring Maintenance of the GSH Pool. *American Journal of Pathology* 160(3): 1115-27
- **West JA**, Pakehham G, Morin D, Fleschner CA, Buckpitt AR and Plopper CG (2001) Inhaled naphthalene causes dose dependent Clara cell cytotoxicity in mice but not in rats. **Cover Page** *Toxicology and Applied Pharmacology* 173:114-119
- Plopper CG, Buckpitt A, Evans M, Van Winkle L, Fanucchi M, Smiley-Jewell S, Lakritz J, **West J**, Lawson G, Paige R, Miller L and Hyde D (2001) Factors modulating the epithelial response to toxicants in tracheobronchial airways. *Toxicology* 160:173-180.

- West, J.A.A., Chichester, C.H., Buckpitt, A.R., Tyler, N.K., Brennan, P., Helton, C., and Plopper, C.G. "Heterogeneity of Clara cell glutathione: A possible basis for differences in responses to pulmonary cytotoxicants." – 2000 – *American Journal of Respiratory Cell and Molecular Biology* 23(1) pp. 27-36.
- West, J., Buckpitt, A., Plopper, C., "Elevated intracellular glutathione (GSH) resynthesis confers protection to Clara cells from naphthalene (NA) injury in tolerant mice" – 2000 – *Journal of Pharmacology and Experimental Therapeutics* 294(2) pp. 516-523.
- Plopper, CG, Van Winkle LS, Fannuchi MV, Evans MJ, Weir AJ, Nishio SJ, Postlewaite ES, West J, Buckpitt AR, Hyde DM. "Basic Principles for Use of Fluorochromes and Filters." *Faseb Journal* 14 (4): A17 Mar 15 2000.

Patents and technical advances:

- *Polymer Microfluidic Biochip Fabrication.* Jesse Thompson, Jay A.A. West. – In Preparation - PCT Patent App. (2007)
- *PCR-free sample preparation and detection systems for high speed biologic analysis and identification.* Kyle W. Hukari, Brent C. Satterfield, and Jay A.A. West, PCT patent App. (2007)
- *Disposable sample preparation cards, methods, and systems thereof.* Brent C. Satterfield, Seth Stern, Michael DeRenzi, Kyle W. Hukari, and Jay A.A. West, US patent App. 60829079 (2006)
- *Cooperative probes and methods of using them.* Brent C. Satterfield, Jay A.A. West. PCT patent App. 60850958 (2006)
- *Selection of Aptamers Based on Geometry.* Brent C. Satterfield, Jay A.A. West. US provisional patent App. 60870493 (2006)
- *Apparatus and method for carrying out chemical or biological processes.* Kyle W. Hukari, and Jay A.A. West, US patent App. (2006) Patent Pending
- *A Sample Preparation Device for Integrated Lyses, Labeling and Analysis of Robust Bacteria, Bacterial Spores, and Viruses.* Jay A.A. West, Kyle W. Hukari, Kamlesh Patel, Ronald F. Renzi PCT2005034045 US60/612,969 (2005)
- *Miniaturized Microfluidic Genearray platform for fluidic handling and real-time detection of array probes.* Jay A.A. West, Kyle W. Hukari, Gary A. Hux (2005) Patent pending
- *High Density Specific Location Microarray Spotting Software.* Kyle W. Hukari, Jay A.A. West. (2004) Copyright pending
- *Viral identification by generation and detection of protein signatures.* Jay A.A. West, Todd W. Lane, James F. Stamps, Isaac R. Shokair, Julia A. Fruetel. US patent App. 20050014134. Patent Pending.(2004)
- *A Modular Device for Microscale Biotxin Detection.* Julia A. Fruetel, Victoria VanderNoot, Brent A Horn, Jay A.A. West, Ronald Renzi, Scott Ferko, James F. Stamps,

Isaac R. Shokair, Daniel Yee, Robert Crocker, Boyd Wiedenman. (2003) US patent App. 20040126279 *Patent Pending*

- *Development of a microfluidic integrated microarray for detection of chimeric biological weapons.* Jay A.A. West, Boyd Wiedenman. (2003) US patent App. 20050095602. *Patent Pending.*

Conference Abstracts, Presentations, and non-refereed articles:

- BioPhalanx I: Development of Diagnostic Reagents to Provide Reliable Detection of Biological Agents Jay A.A. West, Brent C. Satterfield, Matthew Bartosiewicz, Kyle W. Hukari, Jesse Thompson, Fabian Van de Graaf -2008- Annual Chemical and Biological R&D Technologies Conference January 28 – February 1, 2008 – San Antonio, Texas
- Lysix™ MPCs (micro-purification cards) and the Lysix™ Nucleic Acid Workstation 808 Matthew Bartosiewicz, Senior Scientist, Arcxis Biotechnologies Jay West, Ph.D., CTO, Arcxis Biotechnologies, IBC Conference, September 18 - 19, 2007, Philadelphia, PA
- *BIOPHALANX: a hand portable discrete monolithic microarray Biothreat Detector AND Tentacle Probes: discrimination of difficult single nucleotide polymorphisms* Jay West, Arcxis Biotechnologies, Homeland Security Research Innovation & Transition Conference Monterey, CA Aug 21—24, 2007
- Arcxis Biotechnologies: Platforms for Rapid PCR-Free detection of Biological Threats. Kyle W. Hukari, Dustin Li, Mike Derenzi, Seth Stern, Quang Thai, Brent A. Satterfield and Jay A.A. West. Gordon Conference, Chemical & biological terrorism defense, Integrating Biodefense, Homeland Security And Basic Science January 14-19, 2007, Ventura, CA.
- Tentacle probes: High Affinity Reagents for Rapid Discrimination of Single Nucleotide Polymorphisms. Brent A. Satterfield and Jay A.A. West Department of Homeland Security Chemical and Biological Countermeasures conference Oct. 2006 Boulder CO.
- *LYSLX™* Single step sample preparation for the isolation of nucleic acids. Jay A.A. West. National Conference on Environmental Sampling and Detection for Bio-Threat Agents October 25-28, 2006, Brooklyn, NY.
- BioPhalanx, A Portable Discrete Monolithic Microarray Biothreat Detector. Jay A.A. West, Kyle W. Hukari, Brent A. Satterfield, Dustin Li. Bio-Sensors, Assays, and Informatics for Homeland Security” DHS / S&T / HSARPA. Monterey, CA, May 16-18, 2006
- Altix: A Hand-Portable High Density Microarray Platform for Multiplexed Bio-Detection. Jay A.A. West, Kyle W. Hukari, Gary Hux, and Timothy J. Shepodd. Bio-Sensors, Assays, and Informatics for Homeland Security” DHS / S&T / HSARPA. Monterey, CA, May 16-18, 2006
- Integrated sample preparation and analysis of biological agents using the hand portable capillary electrophoresis instrument, μ Chemlab. Jay A.A. West, Kyle W. Hukari, Kamlesh Patel, Ronald F. Renzi. 2004 Proceedings of ECS, Honolulu, HI.
- A High Density Microfluidic Microarray platform for rapid genomic profiling. Jay A.A. West, Kyle W. Hukari, Gary A. Hux. 2004 Proceeding of μ Tas Vol. 1 pp.656-658

- Rapid and complete solubilization of Bacillus spores using a flow through thermolyser for automated sample preparation. Kyle W. Hukari, Ken Patel, Ron Renzi, Jay A.A. West 2004 Proceeding of μ Tas Vol. 2 pp. 321-323
- Rapid Universal Solubilization and Analysis of bacterial spores using a Simple Flow-Through Ultra-High Temperature Capillary Device. 2004 Proceedings of SPIE. Vol. 5591 pp. 10-22, Jay A.A. West, Kyle W. Hukari, Ronald F. Renzi, and Kamlesh Patel
- Microfluidic Gene Arrays for Rapid Genomic Profiling Proceedings of SPIE. 2004 Volume: 5591 pp. 24-36. Jay A.A. West, Kyle W. Hukari, Gary Hux and Timothy J. Sheppard
- Development of a microfluidic microarray for the rapid detection of toxicogenomic signatures. Jay A.A. West, Rachel M. DeVay, Micheal Shultz. Sandia National Laboratories, University of California, Davis. Oral Presentation at the Annual Society of Toxicology meeting 2004. *The Toxicologist* (78) p392.
- Detection of bacterial biowarfare agents with a proteomics-based analyzer V.A. VanderNoot, J.A.A. West, K. Hukari, G.S. Chirica, J. Cintron, J. Fruetel, T.W. Lane, K. Wally, P.F. Caton and B.L. Haroldsen. Sandia National Laboratories (2004) Point detection conference.
- Rapid Detection of Viruses Using μ ChemLabTM, a Hand-Portable Microanalytical Instrument (K3) Jay West, Todd Lane, James Stamps, Isaac Shokair, Ronald Renzi and Julie Fruetel, *Sandia National Laboratories (2004) Point detection conference*.
- Rapid Detection of Pathogens using μ ChemLabTM, a Hand-Portable Microanalytical Instrument. Julie Fruetel, Victoria VanderNoot, Jay West, Todd Lane, James Stamps, Isaac Shokair, Brent Haroldsen and Ronald Renzi. *Sandia National Laboratories, Livermore, California. Gordon Conference on Biological Detection systems. 2004*
- Detection and identification of viral agents by protein fingerprinting using the hand-portable μ Chemlab. Jay A.A. West, Todd W. Lane, James F. Stamps, Isaac R. Shokair, and Julia A. Fruetel *Proceedings of the uTAS 2003 symposium*. 2003. Squaw Valley, USA: Kluwer Academic Publishers.
- Identification of biotoxin variants and viral signatures using the hand portable μ ChemLabTM detection system. Jay A.A. West, Brent A. Horn, Todd W. Lane, and Julia A. Fruetel. Sandia National Laboratories, Livermore CA. Presented at the Annual Society of Toxicology meeting 2003. *The Toxicologist* (72) p164
- Results from μ ChemLab/CB, a portable system for detecting chemical and biological warfare agents. Julia A. Fruetel, Brent A. Horn, Jay A.A. West, James F. Stamps, Victoria A. VanderNoot, Mary Clare Stoddard, Ronald F. Renzi, Sandia National Laboratories, Livermore, CA USA, Debbie Padgen, Defense Science and Technology Laboratory, Porton Down, UK. *Proceedings of the uTAS Symposium*. (vol 1) 524-526
- Engineered improvement of the generation-2 μ ChemLab biotoxin detector. Ronald F. Renzi, James F. Stamps, Robert W. Crocker, Boyd J. Wiedenman, Scott M. Ferko, Brent A. Horn, Dan Yee, Victoria A. Vandernoot, Jason A. West, Julia A. Fruetel, Yolanda Fintschenko, Mary Clare Stoddard. Sandia National Laboratories, Livermore, CA USA. *Proceedings of the uTAS Symposium*. (vol 2) 703-705.

- Phenylethylisothiocyanate (PEITC) both inhibits cytochrome P450 1A1/1A2 bioactivation and induces glucuronide detoxification of the carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). West, J.A., Turteltaub K.W., Dingley K., Lawrence Livermore National Laboratory, Livermore CA. Presented at the Annual Society of Toxicology meeting 2002
- μ ChemLab™: Miniaturized Chemical Analysis System for the Detection of Biotoxins. Julia Fruetel, Ronald Renzi, Robert Crocker, Victoria VanderNoot, Jay West, James Stamps, Isaac Shokair, Daniel Yee. Sandia National Laboratories. Livermore, CA. Presented at the Annual Society of Toxicology meeting 2002
- Phenylethylisothiocyanate (PEITC) reduces 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) DNA adduct formation by inhibition of Cytochrome P450 dependent metabolism. West, JAA, Dingley, K.H., Turteltaub, K.W. Lawrence Livermore National Laboratory, Livermore, CA. Presented at the Annual Society of Toxicology meeting 2002.
- Mechanisms Intrinsic to Clara Cells of Mice Produce Tolerance to Repeated Inhalation Exposures of the Bioactivated Cytotoxicant Naphthalene (NA). Jay A.A. West, Dexter Morin, Henry J. Forman Alan R. Buckpitt, & Charles G. Plopper. Univ. of Calif.-Davis. Presented at the American Thoracic Society Annual Meeting 2001.
- Isolated Clara cells export glutathione (GSH) conjugates by a multidrug resistance associated protein (MRP) transporter. J. West, Z. Johnson, A. Buckpitt, C. Plopper. Univ. of Calif.-Davis. Presented at the American Thoracic Society Annual Meeting 2000.
- Development of naphthalene (NA) tolerance in Clara cells represents an adaptation with few phenotypical changes. J. West, C. Fleschner, E. Toskala, C. Plopper. Univ. of Calif.-Davis Presented at the American Thoracic Society Annual Meeting 2000.
- Clara cell naphthalene (NA) tolerance is independent of hepatic NA metabolism and is associated with increases in glutathione and γ -glutamylcysteine synthetase. J. West, C. Fleschner, D. Morin, H. Forman, C. Plopper, A. Buckpitt. Univ. of Calif.-Davis Presented at the American Thoracic Society Annual Meeting 2000.
- Heat shock proteins are present early in the development of tolerance but appear to play no role in the resistant phenotype. Kurt J. Williams, Jay A.A. West, Chad A. Fleschner, and Charles G. Plopper. Presented (poster) at the American Thoracic Society Annual Meeting 2000.
- Inhaled naphthalene (NA) causes dose dependent Clara cell cytotoxicity in mice. JAA West, G Pakehham, D Morin, CA Fleschner, CG Plopper, and AR Buckpitt. Presented (poster) at the Annual Society of Toxicology meeting 2000
- Elevated intracellular glutathione (GSH) confers protection to Clara cells from naphthalene (NA) injury in tolerant mice. J. West, E. Toskala, V. Wong, A. Buckpitt, C. Plopper. Presented (poster) at 1999 Annual American Thoracic Society Meeting

EXHIBIT B

Reduce Time

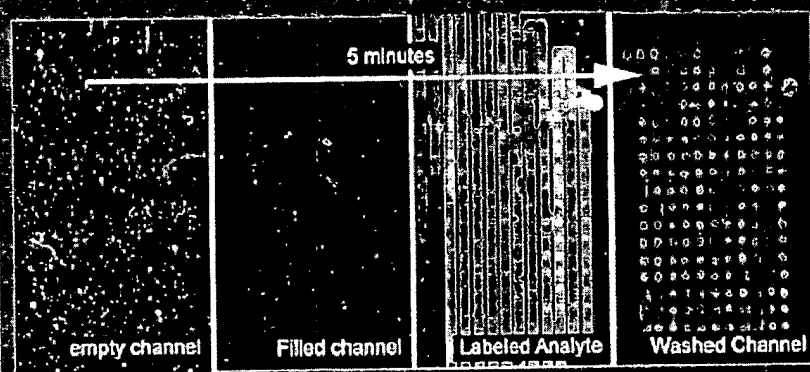
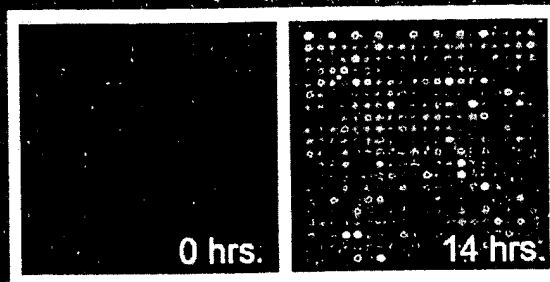


EXHIBIT C

Microfluidic Gene Arrays for Rapid Genomic Profiling

Jay A.A. West, Kyle W. Hukari, Gary Hux and Timothy J. Sheppard
Microfluidics Research Group, Sandia National Laboratories, P.O. Box 969 MS9951, Livermore,
CA. 94551-0969

Abstract

Genomic analysis tools have recently become an indispensable tool for the evaluation of gene expression in a variety of experiment protocols. Two of the main drawbacks to this technology are the labor and time intensive process for sample preparation and the relatively long times required for target/probe hybridization. In order to overcome these two technological barriers we have developed a microfluidic chip to perform on chip sample purification and labeling, integrated with a high density gene array. Sample purification was performed using a porous polymer monolithic material functionalized with an oligo dT nucleotide sequence for the isolation of high purity mRNA. These purified mRNA's can then rapidly labeled using a covalent fluorescent molecule which forms a selective covalent bond at the N7 position of guanine residues. These labeled mRNA's can then released from the polymer monolith to allow for direct hybridization with oligonucleotide probes deposited in microfluidic channel. To allow for rapid target/probe hybridization high density microarray were printed in microchannels. The channels can accommodate array densities as high as 4000 probes. When oligonucleotide deposition is complete, these channels are sealed using a polymer film which forms a pressure tight seal to allow sample reagent flow to the arrayed probes. This process will allow for real time target to probe hybridization monitoring using a top mounted CCD fiber bundle combination. Using this process we have been able to perform a multi-step sample preparation to labeled target/probe hybridization in less than 30 minutes. These results demonstrate the capability to perform rapid genomic screening on a high density microfluidic microarray of oligonucleotides.

Introduction

Sensitive, accurate detection and portable identification of biological agents is critical to our ability to avert wide spread casualties from a bio-terrorist threat. The use of Gene or "micro" arrays has become a widespread bio-analytical technique for genomic profiling. Recent advances in microarray technology have led to diagnostic applications that are currently being developed for the detection of biological pathogens. Two major drawbacks to the current experimental format in which these assay are performed are the degradation sensitive long sample processing time required for probe generation and the extended time requirement for the aforementioned probes to be hybridized to the target DNA on the slide surface. The goal of these studies was to develop an array based microfluidic chip and platform that can be adapted to a hand portable device. We have also focused our efforts to develop a platform that is easily customized for rapid assay development in a wide application space. This will be accomplished by integrating two newly emerging and maturing technologies: microarrays and microfluidics. Microarray devised devices were first described in 1989 by Ekins et. al. [1] where antibodies were affixed to a solid substrate. Subsequent publications Fodor [2] and Schena [3] set the stage for the explosion of *DNA*, *gene* or *Micro* array technology. Since these landmark publications, various chip design platforms including oligonucleotide, EST, and cDNA have been developed along with an ever expanding number sources of *content* or synthesized genes for detection of a particular agent or expressed gene. These array technologies vary widely in their format as well as their application. High density oligonucleotide arrays, based on Fodor's publication [2] later became the base technology for Affymetrix (patent # 5,445,934). These oligonucleotide formats have extremely high density, with the capacity to perform measurements on over 350,000 DNA elements in a single experiment. While this technology has great measurement capacity, due the photolithographic manufacturing process using multiple masks, it is less well suited for integration with microfluidic systems. A better suited microarray platform for microfluidic integration in a custom or robotically spotted cDNA microarray first described by Schena [3].

This array format has demonstrated good correlation to standard quantitative genetic assays such as northern blot analysis [4]. Additionally, using a robotic spotter for array construction offers the capability to deposit high density DNA probes in complex architectures, such as microfluidic systems that also offer the flexibility to routinely alter content of the device to optimize assay conditions rapidly. Low density microarrays have also been recently been developed [5-7]. These devices make use of a plastic microfluidic channel to effect rapid target to probe hybridization times. The current studies were designed to extend this capability to develop on-chip sample purification and rapid sample labeling. We also aimed to produce a device that can perform these rapid hybridization reactions in a high density microarray format.

In order to realistically manipulate this technology for practical purposes outside the laboratory, it is crucial to first determine the most efficient method of hybridizing a target sequence to a known probe sequence. In these experiments, different techniques of labeling mRNA and cDNA, including those with Cy5 dyes and Universal Labeling System (ULS) dyes, were performed. In order to further increase the speed in which these type of analyses can be performed, we have designed and fabricated a microfluidic microarray that will both decrease sample probe preparation time as well as target to probe hybridization time. The development of such devices will allow for the miniaturization of microarray instrumentation and make hand-portable microarray technologies a reality. We first focused on the fabrication of the high density microfluidic microarray that has the ability to detect thousands of individual elements in real-time. We have optimized the design of this chip to affect reduced hybridization times of target oligonucleotides, eliminate diffusion effects to optimize chip-based nucleic acid hybridizations, and to maximize signal to noise ratios in order to reduce the optical limit of detection. We have further developed porous polymer monolith material for the trapping of mRNA. Using this polymer we demonstrate the rapid trapping and release of purified mRNA. To reduce the time required for sample processing, we evaluated several commercial nucleic acid labeling protocols. These experiments demonstrate that it is possible to generate hybridization ready samples in less than twenty minutes. Finally we have developed the first generation fluidic and detection platform. This platform will allow for easy use of the fabricated microfluidic chips, and is capable of both performing fluidic control elements as well as optical detection. We are currently integrating thermo-controlling capability into the device. In this manuscript we demonstrate the individual processes, such as sample trapping and rapid hybridization, in order to translate these technologies to a completely integrated hand-portable device.

Materials and Methods

Reagents and supplies: The following chemicals were used at various steps of experimentation and were used without further purification. 3-Glycidialoxypropyldimethoxymethylsilane, 99% pure Hexanes, Ethanolamine, sodium dodecylsulfate (SDS), NaCl, and Ethanol were purchased from Aldrich Chemical Co. DEPC water, 20X SSC, 20X SPPE buffers were purchased from Invitrogen. Rat oligo test set, spotting buffers, and epoxy coated microscope slides were purchased from MWG. PCR clean-up kits were purchased from Qiagen. The microfluidic manifold was constructed on site using Delrin and aluminum stock. O-rings were purchased from Apple Rubber. Microfluidic fittings made with PEEK were supplied by Sandia National Labs. Ulysis DNA labeling kits were purchased from Molecular Probes (Eugene, OR.). CyScribe kits for first strand synthesis and cytosine labeled Cy5 dyes were purchased from Amersham biosciences. All other chemicals purchased were reagent grade or better.

Microfluidic chip fabrication: Devices were optimized by experimental modeling and fabricated using standard photolithography techniques. Microfluidic chips were fabricated in fused silica which has the advantage of detecting fluorescent spots with a high signal/noise ratio [8]. Fabrication with fused silica allows for the use of multiple etch depths to normalize pressure gradients and reduce the diffusion distance for target analytes to the probe surface. This reduces hybridization time dramatically. Integration of microarrays with a microfluidic device was accomplished by designing and fabricating areas for performing sample clean-up, amplification and/or hybridization on a single chip. The construction of these arrays was accomplished using a multiple depth photolithographic etch process on two separate four inch quartz wafers which were subsequently bonded at 1100°C.

Spotting of oligonucleotide probes to the microfluidic microarray chips is achieved by use of a robotic spotter that deposits the probes on to a custom addressable array. The robotic spotter has micron resolution to position the spotting pens within the microchannels. Further details of robotic spotting are provided in U.S. Patent Application Serial No. 10/701,097, "Microfluidic Integrated Microarrays for Biological Detection", filed November 4, 2003. After spotting, the microfluidic chip is cleaned and prepped following standard procedures (0.1% sarcosine, next 3x SSC, rinse in deionized water, and immersion in ice cold ethanol). Sample oligonucleotide probes, for example commercially-

available rat DNA probes from MWG Biotech (High Point, North Carolina) are attached to the microarray surface according to these processes.

Cye Labeled ULS and Labeled ULS mRNA: To evaluate different methods of analyzing gene expression (mRNA) first strand synthesis (mRNA to cDNA) was executed with and without incorporating a Cy labeled d(CTP). Unlabeled cDNA was made using 1.0 ul 1 mM d(CTP) instead of the Cy dCTP. The reaction mix (Amersham Biosciences) was then added to the mRNA mix at room temperature. After 10 minutes 1.5 ul of reverse transcriptase (RTase) was added to the mixture, and then placed at 42°C for 1 hour. The reaction was stopped by snap cooling on ice for 30 seconds. 1.0 ul of RNase H and 0.5 ml RNase cocktail (A+T1) (Stratagene) was added to degrade the mRNA. The reaction was followed by purification using a PCR clean up kit (Qiagen) with spin columns. The unlabeled cDNA and mRNA were labeled using various Universal Labeling System (ULS) dyes which utilize a platinum complex that attaches to the the N7 position of Guanine in nucleic acids. 1.0 ug of mRNA was added to 19 ul of the labeling buffer and denatured at 95°C. The cDNA was incubated for 15 minutes at 80°C, and the mRNA incubated for 10 minutes at 90°C. Concentrations and base/dye ratios were determined using a microplate reader.

Monolith Development:

The synthesis of suitable probes for microarray analysis depends on the isolation of high quality RNA or DNA samples, efficient labeling of these purified samples, and removal of unbound dye prior to probe hybridization. Using UV initiated porous polymer monolith we are able to selectively pattern nucleic acid purification columns in microfluidic chips. Once polymerized, these monolith columns are post functionalized with a variety of amine terminated oligonucleotides which traps the targeted nucleic acids. Such monoliths are especially well adapted to microfluidic devices, as they can be polymerized in any particular pattern, have a selectable porosity, and high surface area for efficient nucleic acid binding. Trapping of target oligonucleotides was accomplished using a UV cured porous polymer monolith. Chips are pretreated overnight with a 10 parts water, 6 parts acetic acid, and 4 parts z-6030 solution. Chips are then flushed with filtered buffer and dried. To Prepare the monomer solution; add to a 7-mL vial, (added in this order:) 1940 uL of methanol, 660 of ul ethyl acetate, 840 uL of GMA, 560 uL EGDMA, and 8 mg of Irgacure 1800 were combined and vortexed to mix thoroughly. Using a hood vacuum and nitrogen supply, the monomer solution was purged 3 times to remove the oxygen. then sonicated under a vacuum to remove gases. The fluidic chip was filled with monomer solution (flush with at least 10 volumes), taking care to avoid bubble formation. The free monomers in the chip were then polymerized in a UV crosslinking oven for 30 minutes.

Fluidic hardware and detection platform development: Chip manifolds and supporting hardware were designed and fabricated to interface the microfluidic chips using an O-ring face seal. Detection of DNA microarray spots on the microfluidic chip was accomplished using a combination of light delivery using fiber waveguides with a CCD array detection platform for detection of fluorescent spots on the array surface. Using AA battery power to drive an 80mW LED, we are able to deliver light to the chip using a custom fabricated fiber bundle waveguide. This waveguide illuminates the channel where the DNA microarray is located. Oligonucleotide probes were then deposited in the open microchannels using a conventional arraying robot which was controlled by a custom script to allow the deposition of probes in specific locations. These open channels containing the covalently linked oligonucleotides were then sealed with a polymer film in order to flow reagents to each spot on the device. The microarray is sealed using pressure or adhesion with an optically transparent chemically resistant plastic film. The microarray can be adhesively sealed by treating a chemically resistant clear plastic sheet (e.g., PDMS) with plasma oxidation to activate surface functional groups. Amine-terminated silane was then applied to the plasma oxygen activated surface to enable an epoxy-amine sealing reaction between the amine coated surface of the plastic film and the epoxy coated fused silica chip. The plastic surface is then bound to the tops of the ridges between the channels and top side of the chip. Chips are housed in a manifold outfitted with o-ring seals designed to allow for one step chip alignment and to facilitate fluid connections to the outside environment. A Basler A102fc color CCD camera with an 2:1 pulled fiber optic was purchased from Videoscope International. Capillary tubing was purchased from Polymicro, inc.

Results

The microfluidic chips were designed to accommodate on-chip sample purification, labeling and array analysis. To accomplish this, specific regions for sample concentration and arraying are designed into a single device. Using this process we were able to create areas on a compact device (2.5x3.1cm), which can accommodate UV, patterned monoliths for sample processing (figure 1 B&C) and an array-spotting surface for post-fabrication DNA arraying. We designed (figure 1A) and fabricated (figure 1B) the first generation of microfluidic microarrays. In this process, two wafers are etched then, bonded to produce the microfluidic chips. Fabricated Chips (Figure 1B) contained an open channel that is etched 15 μm into the top surface of the microfluidic device (Figure 1B&C). This design allows spotting of DNA probes on the fabricated chip in an open channel. This architecture serves several purposes. Critical to the design of these chips is the ability to robotically spot DNA detection (probes) on the channel array surface after fabrication of the microfluidic chip. The shallow serpentine micro channel (15 μm height x 300 μm wide) greatly reduces the hybridization time by minimizing the diffusion time of the sample and fluidically directing the target past each surface probe. The second set of channels are closed and are located between the two wafers. These channels house a monolith material that is used for sample purification.

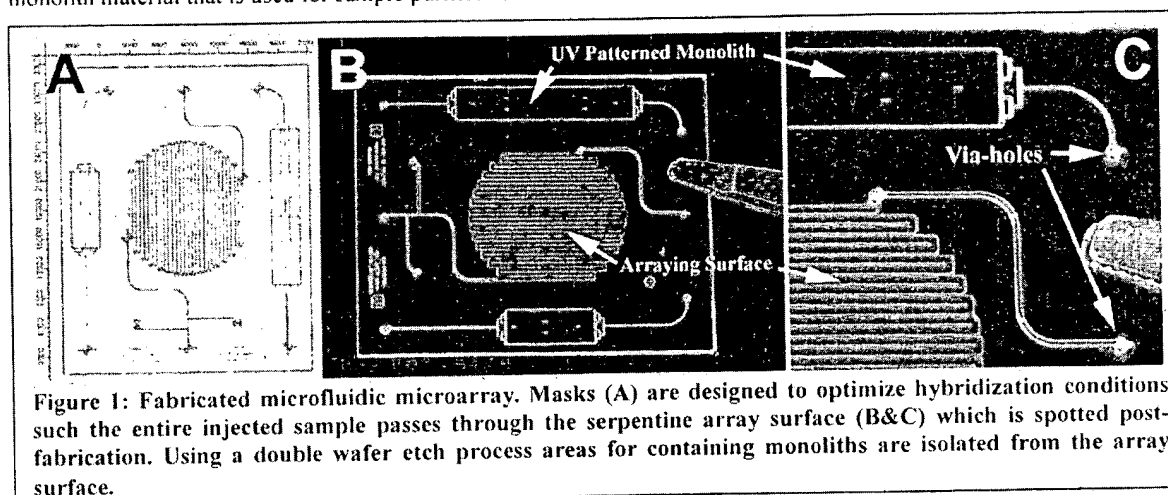


Figure 1: Fabricated microfluidic microarray. Masks (A) are designed to optimize hybridization conditions such the entire injected sample passes through the serpentine array surface (B&C) which is spotted post-fabrication. Using a double wafer etch process areas for containing monoliths are isolated from the array surface.

Monolith: The Oligonucleotide trapping monolith is composed mainly of glycidyl methacrylate (GMA) which has a porous structure with roughly 1-3 μm pores (Figure 2A). The Glycidyl (epoxide) chemistry allows for post-functionalization of the polymerized monolith, as seen in Figure 2B&C. Polymerized monolith (Figure 2B), is not fluorescent at 488nm (blue light). In contrast when this monolith is post-functionalized (C) with any amine containing molecule (Oregon Green), the monolith appears as a fluorescent signal housed in the capillary (Figure 2B).

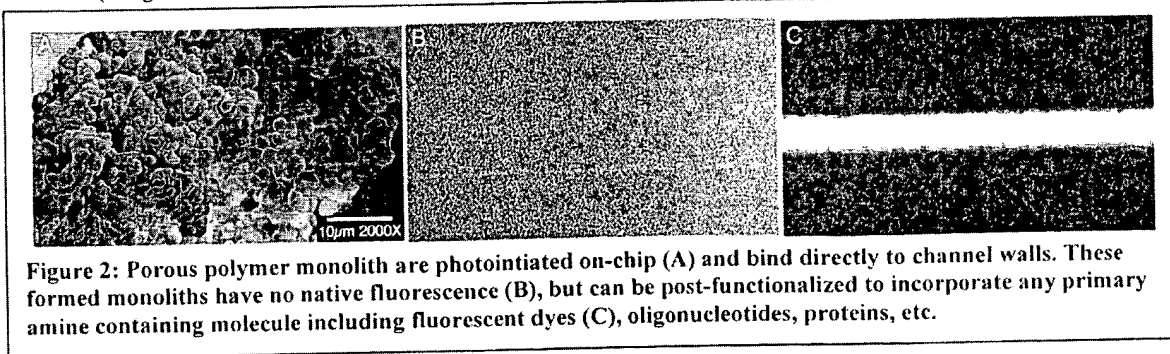
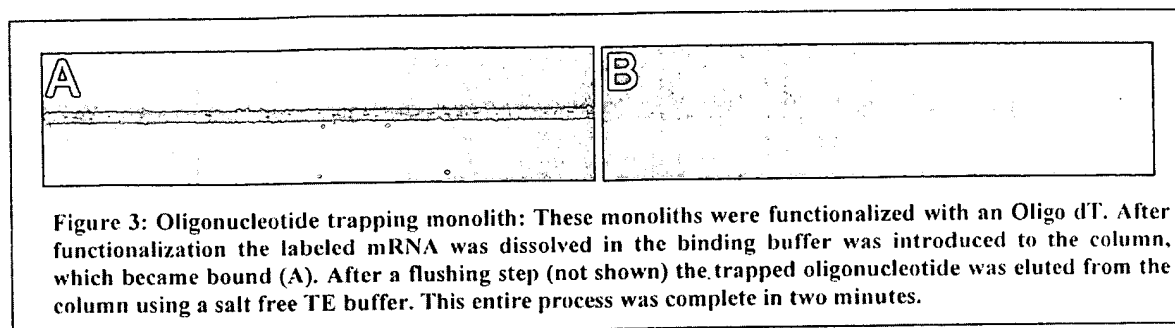
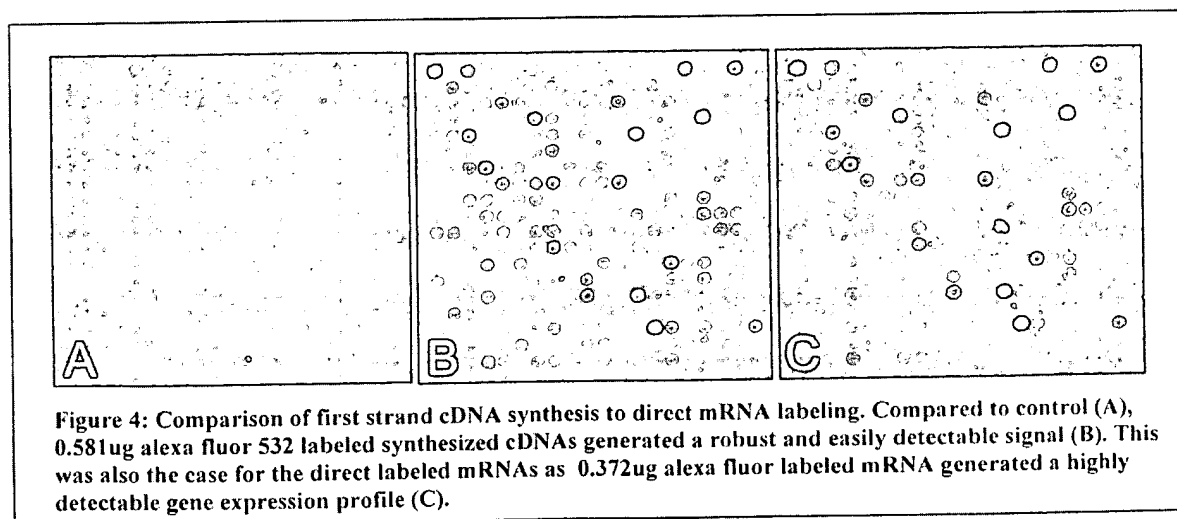


Figure 2: Porous polymer monolith are photoinitiated on-chip (A) and bind directly to channel walls. These formed monoliths have no native fluorescence (B), but can be post-functionalized to incorporate any primary amine containing molecule including fluorescent dyes (C), oligonucleotides, proteins, etc.

In order to trap oligonucleotides the glycidyl methacrylate polymer was functionalized using an amine terminated oligo dT which contained a C6-linker molecule. Once the monolith was polymerized in the channel the oligo dT solution was introduced in a Tris buffer pH 8.3. The monolith was then heated to 60°C for thirty minutes in a humid chamber. Unbound oligo dT was then flushed out using additional buffer solution. mRNA's (in a TE/SSC buffer) were then isolated by using a syringe and syringe pump to deliver flow through the porous polymer monolith. The hybridization in the monolith is rapid, occurring in less than two minutes (figure 3A). After a secondary flushing step using the binding buffer, which does not result in decreased fluorescence, the mRNA was eluted using the TE buffer as seen in Figure 3B.

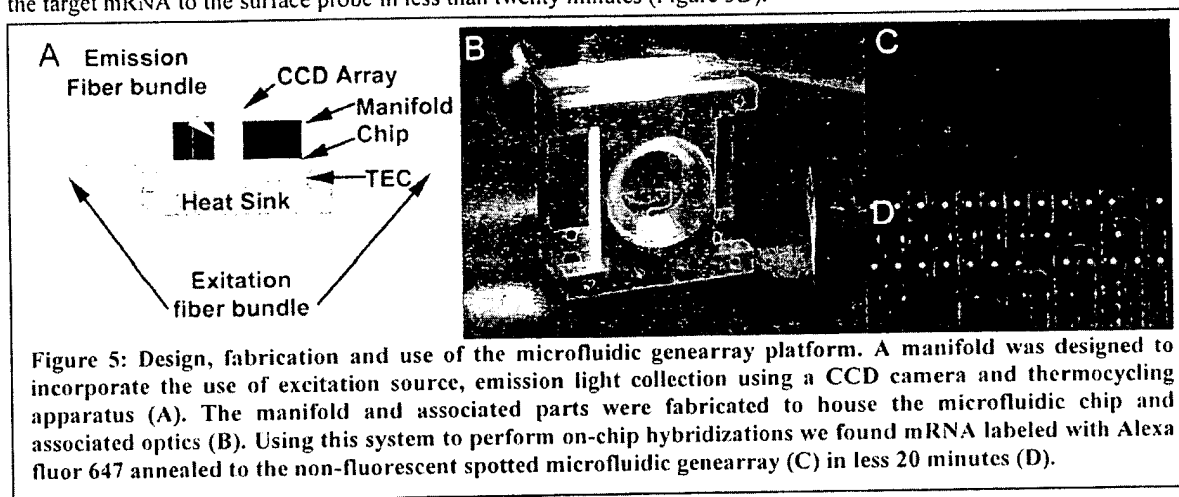


mRNA labeling: To determine the optimal strategy for fluorescently labeling nucleic acids we tested three commercial protocols for use with our system. Depending on the labeling strategy the intensity of the probe hybridization on the microarrays appears to have variability, while the patterns of gene expression appear identical between labeling platforms (data not shown). In general the direct incorporation of Cy5 labeled oligonucleotides produced more consistent labeled cDNA's. However, as previously reported they appeared to be significant bias in the incorporation of the Cy3 vs. Cy5 dye set into the synthesized cDNA's. We also found that this labeling technique is not well suited for the labeling of oligonucleotides for the rapid detection of genetic signatures. In contrast, we found that the direct labeling of mRNA was an efficient technique for obtaining labeled full length nucleic acids. We found that the expression patterns between synthesized cDNAs (figure 4B) and direct labeled mRNA (Figure 4C) were identical. In addition, compared to the first strand (cDNA) method, the direct labeling of mRNA can be accomplished (including clean up) in approximately 10 minutes.



Detection and Hybridization: In order to detect the hybridization of target to probe on the array surface a detection platform that utilizes a second collection fiber bundle array is placed on the array surface (Figure 5A). When the microfluidic chip is illuminated the pattern of the array surface is easily observable through the fiber bundle (figure 5B).

When this collection fiber bundle is mated to a color CCD camera the CCD camera is placed adjacent to the fiber bundle which is placed on the plastic film for imaging the microarray (Figure 5A). Using this system it is possible to detect fluorescent spots on the array surface or presence of fluorescent dyes in solution in the channel. To observe the hybridization of mRNA in the microfluidic microarray chip, the chip was first spotted with oligonucleotides. The chip was then sealed using a compression plug which sealed a polymer film on the fused silica microarray chip. The labeled mRNA was pre-heated to 90°C, then introduced to the array which was held at a constant temperature of 42°C. The mRNA solution was then allowed to stand in the array channel for 5 min, after which addition aliquot of solution was passed into the array. After the second incubation period was complete the channel was imaged. We found the images collected prior to the hybridization (figure 5C) did not display any detectable mRNA target probe hybridizations. In contrast in when fluorescently labeled mRNA were passed through the channel were able to detect the hybridization of the target mRNA to the surface probe in less than twenty minutes (Figure 5D).



Discussion:

Our results demonstrate the feasibility of fabricating high density microfluidic genearray chips with the capability of performing rapid genomic profiling. The chips are simple to use and easily customizable allowing for the rapid alteration of probe content. In addition the entire assembly is miniaturized and can be packaged in a hand-portable platform for field analysis of biological agents. Previous work by Lenigk, et.al demonstrated the feasibility of the production of microfluidic genearrays [7]. In these studies the number of probes was limited to capability of the device used to deposit oligonucleotide probes in the microfluidic channels. In the present study we have developed a custom spotting scripting software program that allows for the deposition of up to 4000 individual elements in selectable positions along the microfluidic channel. As demonstrated in the previous studies [6, 7], the use microfluidics dramatically decreases the time required for the hybridization to the oligonucleotide probes in the microchannel. In our studies we have seen hybridization times of less than twenty minutes. In addition we have developed an integrated platform for the easy use of the microfluidic chips. This system includes the development of sample trapping monolith polymers which have the ability to trap and elute fluorescently labeled mRNA in less than two minutes. These polymers were similar to those reported for use as a separation matrix for polycyclic aromatic hydrocarbons [9-11]. During the fabrication of the polymers in this study we took great care in preserving the glycidyl functionality of the polymerized monomer. After this initial polymerization a second reaction to functionalize the polymer was conducted. We found that we were able to extensively functional the surface of this polymer with any primary amine containing molecule. In this study this was limited to amine terminated fluorescent dyes, and Oligo dT. However, the flexibility of this polymer will make the functionalization of the surface chemistry capable of accepting any selective trapping molecule. Such chemistry will allow for the use of these columns as pre-selection concentrators for enrichment of a subset of genes of interest in following studies.

We further evaluated the ability to rapidly label nucleic acids using a direct labeling method. We found that the direct labeling method held several advantages over the current standard first strand synthesis of cDNAs. First, because

the reaction is simple, we found far fewer difficulties with contamination of labile mRNA samples. In addition, the method was far more rapid, only requiring a maximum of twenty minutes compared to the usual four-six hour procedure required for first strand cDNA synthesis. Finally, we found that the using this enzyme free system was far easier to integrate with our assay platform as the labeling of trapped mRNA samples can be accomplished by only changing a single solution in the polymer monolith.

Detection of the amplified microarray spots can be carried out using optical fiber bundle arrays, that both transmit and collect light. These fiber bundles deliver high intensity light from low power consuming and robust LED's. Detection is carried out with a high resolution CCD array capable of collecting signal from all elements on the microarray in parallel. The resolution of this CCD array makes it possible to detect all spots in parallel without the need for focusing optics. The CCD array/fiber bundle is placed directly onto the arraying surface of the chip (figure 1B). This capability will make it possible to detect the presence or absence of thousands of threat genes in *real-time*.

In summary, we demonstrate a first generation integrated microfluidic high density microarray platform (up to 4000 probes), that has the ability to house rapid sample preparation, hybridization, and detection of genomic samples. This allows sample preparation of less than 40 minutes including; direct labeling of mRNA's, trapping with porous monolith and elution for hybridization. Most importantly this device demonstrates the hybridization of mRNA's in a microfluidic channel in less than 20 minutes. In the future we plan to perform real time detection of probes using a the fully integrated handheld portable device.

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